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Changes in nitrate reductase activity during the growth of the green alga, *Chlamydomonas reinhardtii*

by

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Abstract

Five experiments were performed in which nitrate reductase (NR) activity and cell number were determined daily for 6 to 9 days. NR activity was determined by assaying for nitrite released when the cells were permeabilized with nystatin. Nitrate reductase activity per cell was measured during the growth of the unicellular alga, *Chlamydomonas reinhardtii.* Enzyme activity was found to fluctuate, with activity being highest during the first two days of culture growth, then rapidly decreasing.

Although patterns in enzyme activity were similar among the first 4 experiments, some variability was seen. In Experiment 5, modifications in experimental design resulted in decreased variability. An understanding of the effect of culture conditions on NR activity in *Chlamydomonas reinhardtii* should prove useful in attempting to look for the presence of this enzyme in other organisms, such as *Euglena gracilis.*

Introduction

Nitrate reductase (NR) is a substrate inducible enzyme that is necessary for the growth and metabolism of all plants and most algae. This enzyme is responsible for the first step in nitrogen utilization, following the uptake of nitrate from the surroundings. Before nitrate can be used in metabolism, it must first be converted to a more reduced form. NR is responsible for the reduction of nitrate to nitrite. The enzyme, nitrite reductase (NiR) then performs the conversion from nitrite to ammonium. Next, the ammonium attaches to carbon skeletons forming organic-nitrogen compounds. Examining the factors which affect NR activity during the growth of an organism will enable researchers to better understand nitrogen metabolism and its relationship to carbon metabolism.

NR in the unicellular alga, *Chlamydomonas reinhardtii,* was studied in order to determine patterns of NR activity during culture growth. In addition, a preliminary experiment was conducted to study the effects of acetate on culture growth, NR activity per cell, and the presence of nitrite in the medium. Understanding the fluctuations in the enzyme activity during culture growth will help in assaying other algae such as *Euglena gracilis* for NR.

Nitrate utilization in Euglena. The original purpose of this research was to determine if the unicellular alga, *Euglena gracilis,* contains NR. The current literature contains conflicting reports regarding *Euglena's* ability to use nitrate as a nitrogen source. Some researchers conclude that *Euglena gracilis* can use either ammonium and nitrate compounds, or certain amino acids as nitrogen sources for growth (Buetow, 1968). A

strain of *Euglena gracilis* var. *bacillaris,* has the ability to reduce nitrate, suggesting that this organism contains NR (Buetow, 1968). In addition, there are reports that nitrate and ammonium are comparable nitrogen sources for *Euglena* (Cramer, et. al., 1952). Opposing reports, however, show that in some strains of *Euglena*, negligible growth is seen in cultures using nitrate as the sole nitrogen source (Oda, et. al., 1979). Buetow (1968) cites other articles asserting that potassium nitrate (KNO_3) was found to be a poor nitrogen source for *Euglena's* cell growth. Buetow also suggests that neither NR nor nitrite reductase has been reported in *Euglena* (Buetow, 1968).

Euglena gracilis. This photosynthetic autotroph is evolutionarily distinct because it seems to be one of the few groups of autotrophic organisms in which the presence of NR is questionable. Determining whether NR is present in this alga would provide a better understanding of nitrogen metabolism and utilization in *Euglena,* and would also help us better understand *Euglena's* division in terms of cellular processes such as photosynthesis and chloroplast development (Kindle, et. al., 1989).

NR is thought to be active within the pyrenoid of the chloroplast (Harris, 1989). Since photosynthesis occurs in the chloroplast of a living cell, knowing details about NR would help researchers better understand photosynthesis. Knowing more about *Euglena's* nitrogen utilization would allow various species of *Euglena* to be categorized into metabolic groups (Buetow, 1968). More generally, comparison of characteristics and enzymes from other plants, bacteria, and algae, in which NR is prevalent, will be possible.

Since NR is required for the first step of nitrogen utilization, its absence from *Euglena* would explain reports of *Euglena's* inability to use nitrate as a nitrogen source.

If the division in which *Euglena* is categorized, *Euglenophyta,* does not use NR *Euglenophyta* will be distinctly different from other plant and algal divisions in this respect.

Nitrate reductase. In plant metabolism, nitrate is converted to ammonium in a two-step electron transfer reaction (Kindle et al., 1989) by two enzymes. The first enzyme is NR (Rajeskhar and Oelmuller, 1987). The second is NiR. NR, in conjunction with NADH, reduces nitrate ions to nitrite ions, and is necessary for cellular growth when nitrate is the sole nitrogen source (Kindle et al., 1989).

Nitrate reductase

$$NADH + NO_3^- \longrightarrow NAD^+ + NO_2^- + OH^-$$

NR is an inducible enzyme -- an enzyme whose activity increases in response to change in a specific environmental factor (Filner et al., 1969). Many factors that might affect the inducibility of NR, particularly light and nitrogen sources, have been studied (Campbell et al., 1976). The presence of nitrate in the surroundings stimulates NR (Hopkins, 1995). Synthesis of NR, however, is inhibited by ammonium in many systems (Reiss, 1994).

NR is the most thoroughly studied enzyme which is known to be substrate induced in higher plants (Filner et al., 1969). It is uncertain whether NR is always induced by the presence of the combination of nitrate and light; however, it has been determined that nitrate induces synthesis and is required for enzyme activity. Depending on the algal cell's growth conditions, NR can be found in both active and inactive forms.

The *nit-A* mutant of *Chlamydomonas reinhardtii* contains an altered and inactive form of NR. Inactive NR is not, however, present only in mutant strains. Inactive NR can be assayed and the determination of its presence would be an interesting model system for the study of inducible enzymes (Hopkins, 1995).

The second enzyme in the reaction, NiR, is responsible for the further reduction of nitrite ions to ammonium. In eukaryotes, both NADH and NADPH act as a source of electrons for the reduction process for both enzymes (Campbell et al., 1976). Both nitrate and nitrite reductases are commonly associated with electron-transfer pathways in green cells.

Nitrate assimilation. NR is the key enzyme in nitrate assimilation (Solomonson and Barber, 1989). There are three fundamental steps in the nitrate assimilation process occurring in green unicellular alga (Figures 1): 1) the uptake of nitrate, 2) the reduction of nitrate to ammonium catalyzed by NR and NiR, and 3) the incorporation of ammonium to carbon skeletons (Vega and Menacho, 1990). When nitrate is the main nitrogen source available in the medium, the nitrogen assimilatory process begins with in the transport of nitrate by the active transport system (Galaván et al. 1991). After the entrance of nitrate into the cell, the conversion of nitrate to ammonium occurs. The enzymes glutamine synthetase and glutamate synthetase are responsible for the steps in the incorporation of ammonium into carbon skeletons (Azuara and Aparicio, 1984).

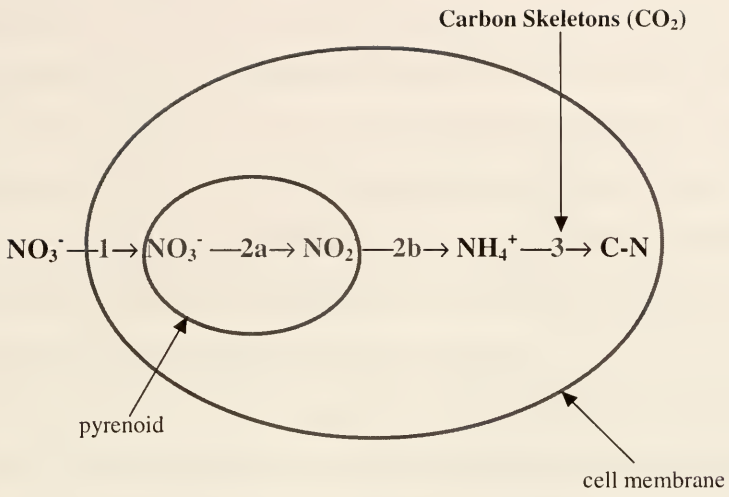


Figure 1: *Diagram of the fundamental steps of nitrate assimilation: 1) the uptake of nitrate, 2a) the reduction of nitrate to nitrite, 2b) the reduction of nitrite to ammonium, and 3) the incorporation of ammonium to carbon skeletons to form organic-nitrogen compounds.*

Nitrate uptake into the cell is controlled by the presence of ammonium, carbon and nitrogen in the medium, and by light availability (Fernández and Cárdenas, 1989). The process of nitrate uptake is almost immediately followed by the reduction of nitrate. As mentioned earlier, it has been found that nitrate is important for the presence and maintenance of NR, but not necessarily the enzyme's activity. In all higher plants and some fungi, the absence of nitrate may cause the cell to contain inactive NR (Hipkin et al., 1980). The enzyme, however, is still considered inducible by nitrate.

Enzymatic assay. Since NR is a substrate inducible enzyme, cells to be assayed for NR must first be grown on nitrate as the sole nitrogen source. NR activity is routinely

assayed by measuring the formation of the product nitrite. Nitrite reacts with sulfanilamide and NEED to form an azo dye, which can then be read spectrophotometrically at a wavelength of 540nm. In working with higher plants, the assay is usually performed in tissue homogenates in which the enzyme has been released by breaking the cells open. With single-celled organisms, an alternative procedure is an *in vivo* test involving the permeablilization of the cell membrane that allows nitrite to leave the cells. This test has advantages in that it is simple and one does not have to be concerned with the actual breakage of and damage to the cells.

An *in vivo* assay has been developed for the single-celled alga, *Chlamydomonas reinhardtii.* The *in vivo* NR assay developed for *Chlamydomonas* uses the antibiotic nystatin to permeabilize the cell membrane. By the addition of nystatin to an aliquot of cells, the cell membrane becomes leaky causing nitrite to be released (Kindle, et. al., 1989). In addition to nystatin, other techniques for nitrite release have been used on unicellular algae. These include: toluene, acetone, liquid nitrogen, freezing and thawing, breaking of cells by the use of a sonicator, and grinding of cells in a cold mortar with alumina (Fernández and Cárdenas, 1982).

My preliminary research consisted of looking for the presence of NR in *Euglena.* Unfortunately, an assay for detecting NR activity has not been developed for this alga. In order to test for NR in *Euglena,* an *in vivo* enzyme assay first had to be perfected in another organism having similar characteristics for which the assay has been designed. This led me to my work with *Chlamydomonas.*

Chlamydomonas. Nitrate utilization, NR, and the organism's life cycle have been thoroughly studied in *Chlamydomonas reinhardtii. Chlamy* is the algal species of choice

for a variety of studies, primarily genetic analysis and maternal inheritance (Harris, 1989). The habitat of the genus is diverse and worldwide. The genus *Chlamydomonas* can be found in most fresh waters. It is characterized by its polar structure, paired apical flagella, and a basal chloroplast surrounding one or more pyrenoids. The pyrenoid is the principal locus of NR. One ability of *Chlamydomonas* is to grow on an organic carbon source in either light or darkness is also known. However, the effect of organic carbon sources on NR activity has not been studied.

Biochemistry of NR. Nitrate reductase is a complex enzyme considered to be the limiting step in nitrate assimilation (Lu, 1994). It is a soluble, dimeric, multicenter redox enzyme (Campbell et al., 1976). NR can be divided into three main functional parts or redox centers: 1) molybdenum, 2) cytochrome b_{557}, and 3) flavin adenine dinucleotide (FAD) binding domains (Lu, 1994). The electron transport chain of nitrate reductase has been suggested as (Solomonson and Barber, 1989):

$$NAD(P)H \rightarrow (FAD \rightarrow \text{cyt b-557} \rightarrow Mo) \rightarrow NO_3^-$$

The location and structure of NR has been extensively studied in both higher plants and algae. In *Chlamydomonas*, the enzyme has been located in the pyrenoid of the chloroplast (Fernández and Cárdenas, 1989). The assimilatory NR molecule in *C. reinhardtii* is heteromultimeric and has a molecular weight of 220 kDa with two kinds of subunits: 1) diaphorase, and 2) terminal. However, the total number of subunits is unknown (Solomonson and Barber, 1989). The diaphorase subunit is responsible for mediating electron transfer *in vitro* from NADH or NADPH to oxidize acceptors. *In vivo*,

this reducing power is transferred to the terminal nitrate reductase to produce nitrite (Harris, 1989).

Types of nitrate reductase. In higher plants, two types of nitrate reductase have been isolated and characterized: 1) NAD(P)H: NR, and 2) NADH: NR. In eukaryotic, single-celled green algal cells, there are three related forms of nitrate reductase: 1) one specific for NADH (NADH:NR), 2) one that operates with NADH and NADPH, and 3) one that is specific for NADPH. NADH: NR is the most common enzyme form in higher plants and algae. NADH: NR is a multimeric enzyme of high molecular weight containing several electron transport components (Azuara and Aparicio, 1983).

Factors affecting NR activity. Factors that affect NR activity include: 1) nitrate, 2) carbon skeletons, and 3) ammonium. Carbon dioxide availability has also been found to affect nitrate uptake and nitrate reductase activity. Azuara and Aparicio studied the effect of nitrate concentration on the consumption and excretion of nitrite and ammonium in *Chlamydomonas reinhardtii*. It was found that exposure to air and low concentrations of nitrate did not affect the excretion of nitrite and ammonium, whereas CO_2-free air did (Azuara and Aparicio, 1984). This is evidence of an important relationship between the carbon and nitrogen metabolisms of *Chlamydomonas*.

Ammonium is another factor that affects nitrate assimilation and NR activity. NR activity is typically low or negligible when ammonium is present, and it is usually at its highest when nitrate is the sole nitrogen source in the medium (Hipkin et al., 1980). In higher plants and algae, the addition of ammonium to nitrate grown cultures causes the deactivation of NR. In *Chlamydomonas reinhardtii,* ammonium inhibits nitrate uptake by blocking the transport process, therefore causing the inactivation of the enzyme's

synthesis and activity (Fernández et al., 1982, Franco et al., 1988). Ammonium can also reversibly inactivate NR (Vega and Menacho, 1979).

Importance of carbon skeletons. As previously mentioned, the nitrogen assimilatory process reduces nitrate to ammonium which then attaches to carbon skeletons for the synthesis of organic nitrogen compounds (Azuara and Aparicio, 1984). A sufficient amount of carbon skeletons are required for the completion of nitrogen assimilation. Research suggests that in *Chlamydomonas,* when carbon dioxide is limited, both nitrate uptake and nitrate assimilation are affected (Vega and Menacho, 1990). It appears that a limited availability of carbon skeletons in the medium during cellular growth causes the excretion of nitrite and ammonium by the cells. It is possible that by increasing the concentrations of organic carbon in the medium, the ammonium will have sufficient carbon skeletons on which to bind, minimizing the release of large amounts of nitrite into the medium.

Azuara and Aparicio noticed that when CO_2 was dissolved in the medium at low cell densities, there was an abundance of carbon sources available for the allocation of all reduced combined nitrogen. At high cell densities, there is an insufficient number of available carbon skeletons (Azuara and Aparicio, 1984). The literature shows that insufficient levels of carbon dioxide deprive the cells of required carbon skeletons to which the ammonium and nitrite bind. This results in the excretion of nitrite and ammonium into the medium. In *Chlamydomonas,* it is unknown whether the addition of organic compounds or an increase in the concentration of organic compounds will substitute for CO_2 requirements. The effects of this variation on nitrite excretion and on NR activity are unknown.

Acetate as an organic carbon source. *Chlamydomonas reinhardtii* can grow with acetate as its sole carbon source; this organism is generally grown mixotrophically (in the light with added acetate). Acetate is thought to be the limiting factor for the growth of *Chlamydomonas.* Because of this, non-photosythetic mutants of *Chlamydomonas* have been grown on media containing acetate and then studied extensively to elucidate the details of photosynthesis (Harris, 1989). Sager and Granick (1953) found that in *Chlamydomonas,* when acetate was added for heterotrophic growth, the medium had a poor buffering capacity, causing an increase in pH from 6.8 to 8.4 (Harris, 1989). However, if nitrate uptake is coupled with hydrogen ion uptake, nitrate uptake would be expected to decrease as the pH increases. This would suggest that increasing acetate would decrease nitrate uptake.

A more detailed knowledge of NR and its activity will provide useful information for better understanding nitrogen utilization and the relationship between nitrogen and carbon metabolism. Understanding the trends in NR activity in *Chlamydomonas* will provide information that can be used in assaying other organisms for the presence of NR.

Materials and Methods

Source of algal cells. *Chlamydomonas reinhardtii,* UTEX #89, was obtained from The University of Texas Culture Collection of Algae.

Maintenance of stock cultures. All cultures were maintained axenically at room temperature (23-25 °C). Stock cultures were kept on a Lab-Line Orbit Shaker at 150 rpm under 6 cool white fluorescent light bulbs 32cm from the culture. Irradiance was 32 μmol $s^{-1}m^{-2}$. Stocks were maintained by transferring approximately 1ml of culture from the previous flask into a 125ml Erlenmeyer culture flask containing approximately 50ml of medium. Transfers were carried out under sterile conditions using a laminar flow hood. Each culture flask was transferred weekly in duplicate.

Chlamydomonas was grown under conditions similar to those described by Reiss (1994) and Kindle et al. (1989). The cell stocks were grown mixotrophically on SGII-NO_3, a modified version of Sager-Granick medium II (Appendix A). The modified version contains increased phosphate, and KNO_3 instead of NH_4NO_3 as a nitrogen source. The cells grown for testing were transferred in aliquots of 0.25 ml, or 0.50 ml, depending on the particular eperiment, into 50 ml growth medium.

Growth of experimental cultures. 0.25ml aliquots of stock culture were transferred to 50ml SGII-NO_3 mixotrophic growth medium (Appendix A) and maintained in the light at room temperature (23-25 °C). These cultures were kept stationary under

two 30 watt cool white fluorescent light bulbs 49cm from the culture. Irradiance was 32 μmol $s^{-1}m^{-2}$; however, for Experiment 4B, irradiance was 17 μmol $s^{-1}m^{-2}$.

Cell counts. Cells were immobilized by adding an 80μl aliquot of cells to 20μl of Lugol's Iodine (1:10) in an eppendorf tube. For calculation of cell densities up to about 1 x 10^6, all 25 larger grids of a hemacytometer (each 0.04mm^2) were counted. For greater densities, five of the large grids were counted diagonally and multiplied by 5 to determine the density of the original culture. Each culture was counted in duplicate.

NR assay. Two 10ml aliquots of cells were placed in separate glass conical centrifuge tubes (in duplicate) labeled 0 minute and 10 minute, respectively. The cells were centrifuged at 180 x g for 10 minutes. After centrifugation, the supernatant was removed and saved for the nitrate assay and pH determination.

The pellet was re-suspended in 2ml of nitrate buffer and the tubes were then vortexed. Fifty microliters of 5000units/ml nystatin was added to each of the tubes. They were vortexed vigorously for one minute, and 2ml of 1% sulfanilamide was immediately added to the 0 minute tube. The second tube was allowed to stand for 10 minutes, then 2ml of 1% sulfanilamide was added. Two milliliters of 0.2% N-1-naphthylethylenediamine dihydrochloride (NEED) was then added to both tubes. The samples were left for 5 minutes to allow development of a pink azo dye. After 5 minutes, the cells were sedimented in a clinical centrifuge at 933 x g for 3 minutes.

The supernatant was pipetted off into individual cuvettes. For each sample, an absorption spectrum was run. The absorbance was determined at 540nm in a Beckman

DU-640 Spectrophotometer. A wavelength of 540nm was used due to the suggestions of Reiss (1994); in addition, an absorption spectrum was run to determine the best wavelength. A 2ml aliquot of SGII-NO_3 medium treated with sulfanilamide and NEED, was used as the blank. Ten milliliter aliquots, unless specified, were tested using the enzymatic assay.

Experiments. A series of five experiments were performed in which the cell number, NR activity, and levels of nitrite present in the medium were determined during culture growth. Cultures were grown from aliquots of 0.25ml inoculated into 50ml of the desired growth medium. In each experiment, each culture was started with equal volumes of inoculate (0.25ml) from the same stock culture on day 0.

Experiment 1-3: Six flasks were used in each experiment. The flasks were inoculated with 0.25ml of a 7-day-old stock culture. A different stock culture was used for each of the 3 experiments. Each of the 6 flasks was assayed for NR activity and cell number on a different day. For example, on day 1, Flask 1 was assayed and cell number was determined; on day 2, Flask 2 was assayed and cell number was determined, and so on. Cultures were monitored for six days.

Experiment 4: Two sets of single flasks (Series A and B) were used. Series A and B each consisted of six experimental flasks which were inoculated with 0.25ml from a single 15-day-old stock culture. Each of the 12 flasks was assayed for NR activity and cell number on a different day. For example, on day 1, Flask 1A and Flask 1B was

assayed and cell number was determined; on day 2, Flask 2A and Flask 2B was assayed and cell number was determined, and so on. Light intensity was measured after the experiment was completed. Cultures were monitored for 6 days.

Experiment 5: Two sets (Series A and B) of single flasks were used. Series A and B each consisted of nine experimental flasks which were inoculated with 0.25ml from a single 5-day-old stock culture. Each of the 12 flasks was assayed for NR activity and cell number on a different day. For example, on day 1, Flask 1A and Flask 1B was assayed and cell number was determined; on day 2, Flask 2A and Flask 2B was assayed and cell number was determined, and so on. Light intensity was measured after the experiment was completed. Six additional flasks were maintained solely for observation of cell number. All 27 flasks were rotated daily to make certain that each received similar light intensities. Cultures were monitored for 9 days.

Experiment 6: A preliminary experiment was carried out to test the effect of acetate concentration of NR activity. Three sets of eight experimental culture flasks were used. Flasks were not set up in duplicate. All 24 culture flasks were inoculated with 0.25ml from a single 10-day-old stock culture. Cells were grown on a modified SGII-NO_3 medium containing one of three different amounts of acetate: 0g acetate, 1g acetate (half the normal amount), and 4g acetate (twice the normal amount of acetate). On each day of the experiment, cell number, NR activity, and the presence of nitrite in the medium were determined in one flask in each series. All flasks were rotated daily to make certain that each received similar light intensities. Cultures were monitored for 8 days.

Standard Curve. Known concentrations of nitrite (Appendix F) were assayed by the addition of 1% sulfanilamide and 0.2% NEED. Absorbance was read at 540nm in a Beckman DU-Spectrophotometer. The nitrite standard curve obtained (Figures 1) showed a linear relationship between A_{540} and nitrite concentration.

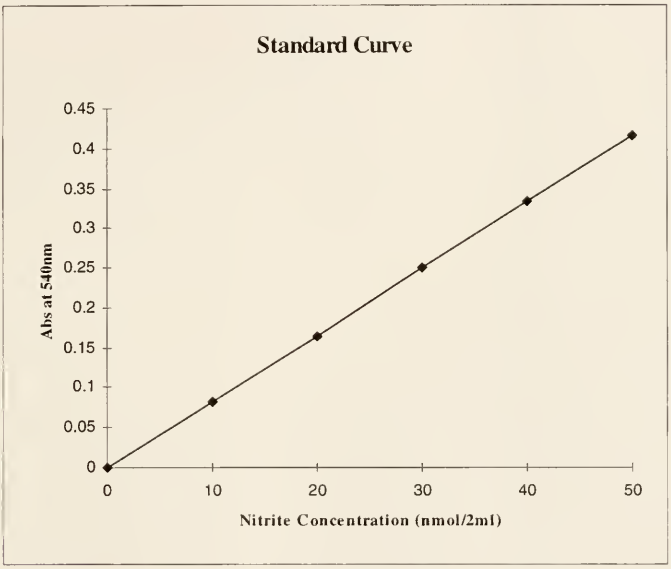


Figure 2: *Standard curve of nitrite concentration determined at an absorbance of 540nm. Refer to Appendix F for protocol.*

Calculation of nitrite concentrations. The 0 minute test tubes served as baselines for samples. In each assay, the absorbance value obtained from the 0-minute tube was subtracted from the absorbance of the 10-minute tube to obtain change in absorbance over a period of 10 minutes. Using this absorbance value, the nitrite concentration for the sample was determined from the standard curve.

Measurement of light. In Experiment 4, irradiance of light ($\mu mol\ s^{-1}\ m^{-2}$) on the cultures was measured with a LI-COR meter equipped with a quantum sensor.

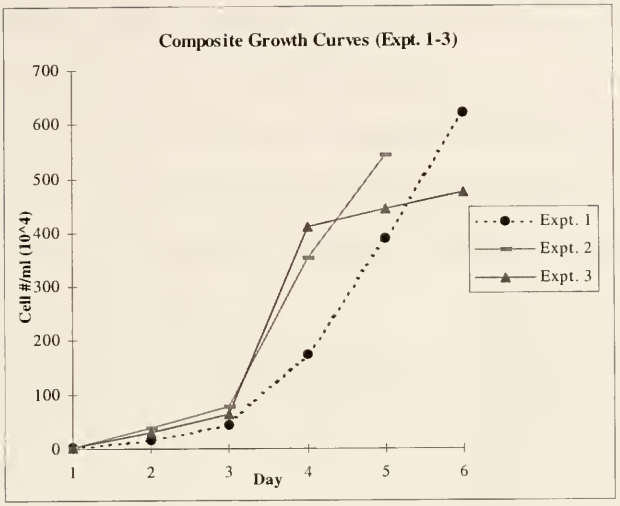
Results

A total of five experiments were carried out to determine the level of NR activity during the growth of *Chlamydomonas reinhardtii*. In the first three experiments, NR activity per cell generally declined with increasing culture age. Because variability was seen among these experiments, duplicate flasks were used in Experiments 4 and 5. In the fourth experiment, uneven light intensity and differences in the age of the stock culture still resulted in much variation. The fifth experiment was designed to better control the variables found in the previous experiments. This resulted in decreased variability, although some small differences were still seen.

Experiment 1-3:

In each of the first three experiments, 6 experimental flasks were used. On day 0, the flasks were inoculated with 0.25ml of a 7-day-old stock culture. A different stock culture was used for each of the three experiments. On each subsequent day of the experiment, a different flask was assayed for cell number, NR activity, and nitrite levels in the medium. For instance, on the first day, Flask 1 was assayed; on day 2, Flask 2 was assayed, and so on. Thus, the growth curves presented here are composites. Each data point represents values from a different flask (Figure 3). A typical growth curve from my experiments has been graphed on a semi-logarithmic plot in order to indicate whether or not the growth curves are exponential. A perfectly linear graph would suggest exponential growth.

Growth curves. The composite growth curves for the three experiments were similar in that the lag phase lasted to day 3. Differences between the experiments were seen in the length of the logarithmic phase and final cell number attained. The log phase began on day 3 and lasted to day 6, with the exception of the third experiment. In Experiment 3, stationary phase was reached on the fourth day. Variability was observed in cell number after the third day of the experiment, in particular on day 6. In this series of experiments, cell number on day 6 ranged from 473 x 10^4 cells/ml (Experiment 1) to 2000 x 10^4 cells/ml (Experiment 2).



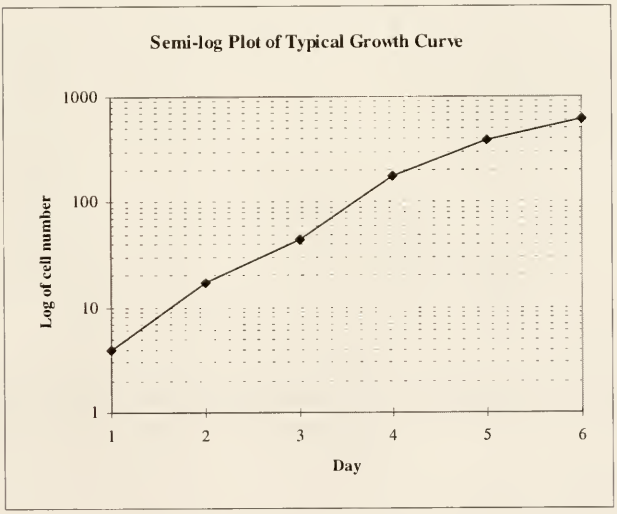


Figure 3: *Composite growth curves for Experiment 1-3. In Experiment 2, the final value for cell number of 2000 x 10^4 cells/ml was not included. In addition, a semi-log plot of a typical growth curve has been plotted to show whether or not culture growth is exponential.*

NR activity. In all three experiments, NR activity per cell decreased dramatically from day 3 to day 6 (Figure 4). The greatest variability in NR activity per cell was observed in the first two days. In Experiment 1, negligible enzyme activity was detected per cell for the first two days, while in Experiments 2 and 3, levels of NR were high. NR activity per cell on day 1 ranged from 0 (Experiment 1) to 22.5 x 10^{-6} nmol/10min/cell (Experiment 3).

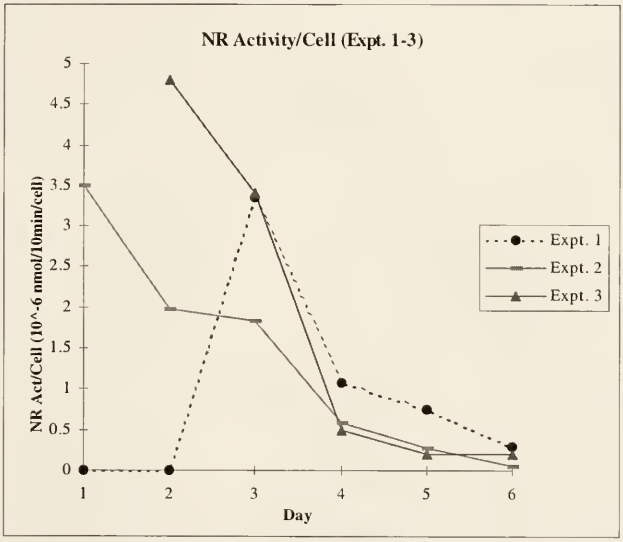


Figure 4: *NR activity per cell for Experiment 1-3. In Experiment 3, the initial value of 22.5 x 10^{-6} nmol/10min/cell has been removed from day 1.*

During the third day of growth in Experiment 1, there was a sharp rise in enzyme activity per cell. The enzyme activity per cell then dropped off at day 4. Thereafter, it slowly decreased during the culture's logarithmic phase of growth.

In Experiment 2, the enzyme activity per cell began high at a value of 3.5 x 10^{-6} nmol/10min/cell. It then decreased rapidly. In the third experiment, NR activity per cell on day 1 had an unusually high value of 22.5 x 10^{-6} nmol/10min/cell. On the second day, the value dropped off. During the log phase of the culture, enzyme activity slowly declined. Once again, much variability is observed between experiments (Figure 5).

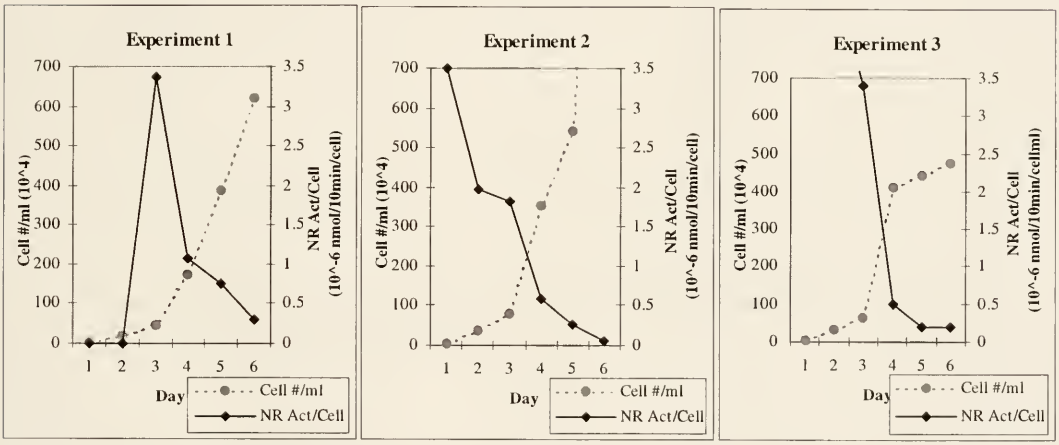


Figure 5: *Composite growth curves and NR activity per cell for Experiment 1-3. In Experiment 2, the final cell number value has been eliminated; in Experiment 3, the NR Act/cell value for day 1 has also been eliminated.*

Nitrite Concentration in the Medium. Small amounts of nitrite were present in the medium, with slight variation, until day 5. From day 5 to day 6, there was a large increase in nitrite in the medium in two of the experiments (Figure 6). On day 6, there is a large quantitative difference in the amount of nitrite present in the medium between the

experiments. In this series of experiments, nitrite values in the medium ranged from 2.1 x 10^{-6} nmol/ml in Experiment 3 to 7.9 x 10^{-6} nmol/ml in Experiment 2 (Figure 6).

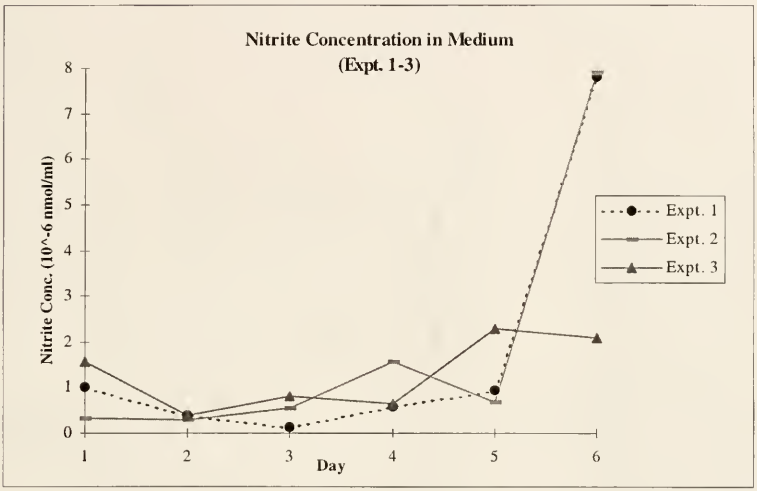


Figure 6: *Amount of nitrite present in the medium during culture growth. Nitrite concentration is expressed as 10^{-6} nmol/ml.*

When comparing graphs of cell number, NR activity per cell, and the presence of nitrite in the medium of Experiment 1-3, several trends become apparent (Figure 7). As cell number increases, the concentration of nitrite in the medium increases, and NR activity per cell decreases. In Experiment 1, cell number is at about 10% of its maximum value when culture growth reaches the end of its lag phase and at the beginning of its log phase. In Experiment 1 and 2, the amount of nitrite present in the medium is also at about 10% of its maximum value until day 5. In just one day, the amount of nitrite in the medium and cell number increase ten-fold. NR activity per cell decreases to about 30%

or less of its initial value by the time the cultures are in early to mid log phase. After NR activity decreases, the amount of nitrite present in the medium then increases to its highest value on day 6.

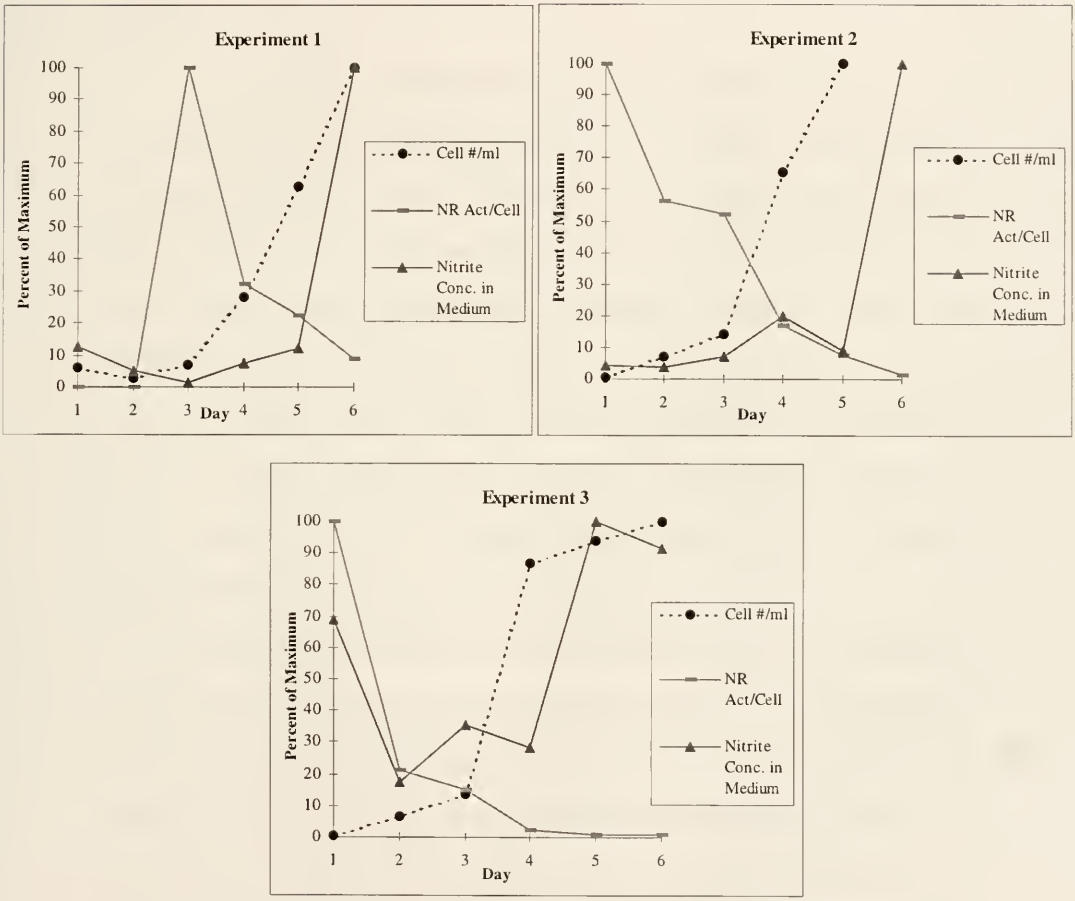


Figure 7: *Comparison of composite growth curves (10^4cells/ml), NR activity per cell (10^{-6} nmol/10min/cell), and amount of nitrite present in medium (10^{-6} nmol/ml) from Experiment 1-3. These values have been adjusted so that 100% represents its maximum value.*

Experiment 4:

Since duplicate flasks were not used in Experiment 1-3, it was not possible to determine the level of variability within an experiment. To correct this problem, an additional experiment (Experiment 4) was set up using duplicate flasks for each day of the assay.

In this experiment, two sets of flasks (Series A and B) were used. Series A and B each consisted of 6 experimental flasks which were inoculated with 0.25ml from a single 15-day-old stock culture. Series B was intended to feature duplicate flasks of Series A. From each of these flasks, cell number was determined daily. Certain flasks were assayed for NR activity, and nitrite concentration in the medium. For instance, on day 1, cell number, NR activity, and nitrite concentrations were determined in Flask 1; on day 2, Flask 2 was assayed, and so on. Light intensity was measured after the experiment was completed. Although all flasks were grown under the same lights, Series A flasks were in a slightly different position and were, thus, inadvertently exposed to twice the irradiance as Series B flasks (Series A: 32 μmol s^{-1} m^{-2} vs. Series B: 17 μmol s^{-1} m^{-2}).

Culture growth. Cell number was counted daily in all flasks. A growth curve from a single flask from each series was determined (Figure 8). A composite growth curve for each series was determined (Figure 9) in which NR activity per cell was also measured. Within a series, growth of the culture was essentially identical in number until day 3. On each day thereafter, cell number in Series A was higher than cell number in Series B. In Figures 9 and 10, lag phase lasted to day 3 or 4. The log phase then began slowly and continued to the end of the experiment.

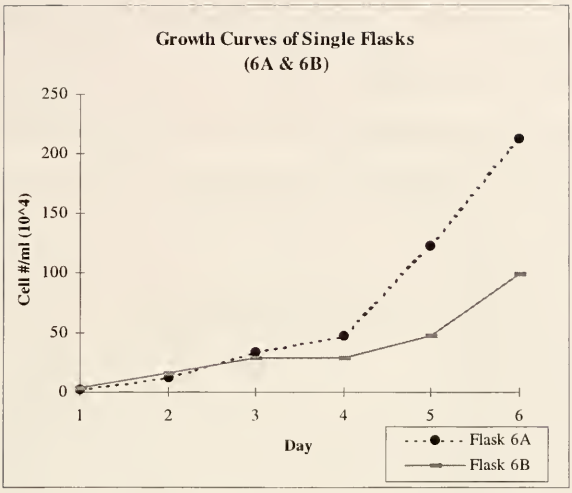


Figure 8: *Growth curves from Series 4A (flask 6A) and Series 4B (flask 6B). Cell number per milliliter (10^4) was calculated daily in each flask.*

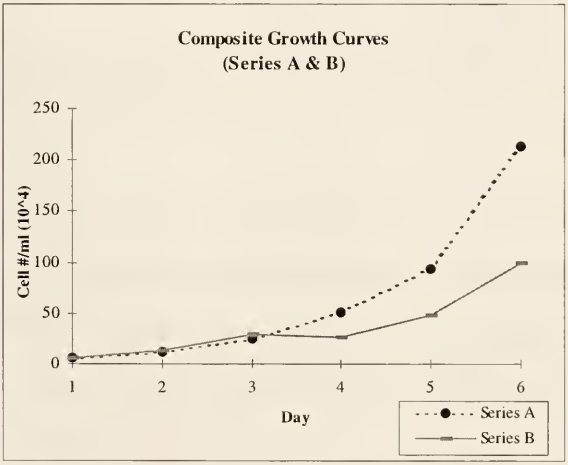


Figure 9: *Composite growth curves from Series 4A and 4B. Cell number per milliliter (10^4) was calculated daily in each assayed flask.*

NR activity. Series A and B from Experiment 4 were similar in that there was no clear pattern of NR activity to day 5. Thereafter, the enzyme activity decreased (Figure 10). Series A began to decrease on day 4, whereas Series B began to decrease on day 5. This difference could be due to large differences in cell number between the two series.

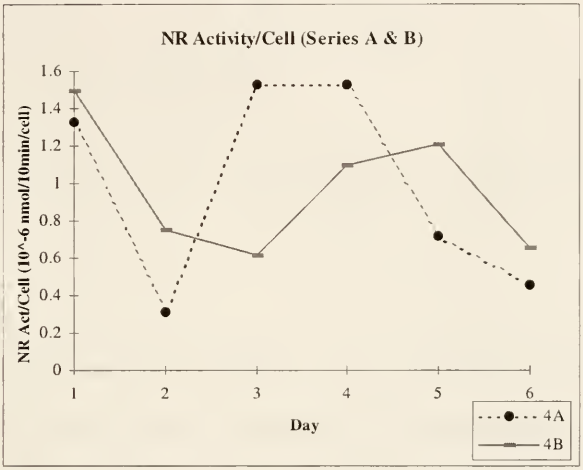


Figure 10: *NR activity per cell in Experiment 4, Series A and B. Enzyme activity is measured in units of 10^{-6} nmol/10min/cell.*

In Experiment 4A, on the first day of the assay, there is more enzyme activity per cell than the last day of the assay; a quantitative difference of almost four times. This is true for Experiment 4B. In both cases, enzyme activity increases during the log phase of growth, and then begins to decrease. In the two experiments, there is much variability in NR activity per cell (Figure 11).

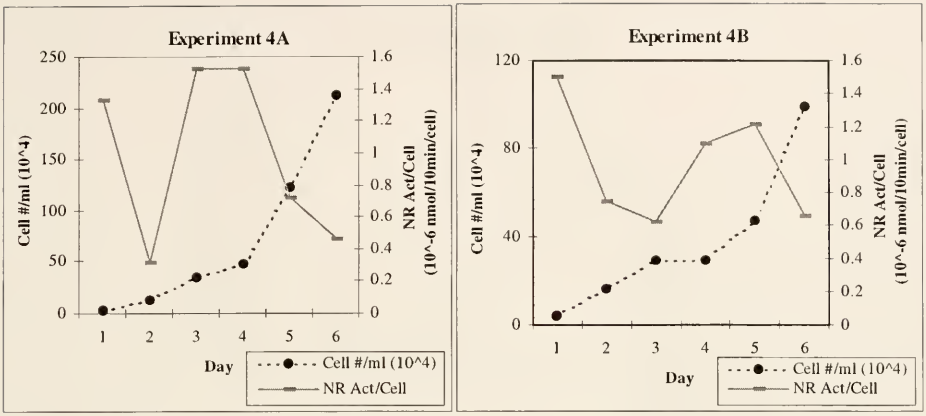


Figure 11: *Composite growth curves and NR activity per cell versus day of assay in Experiment 4, Series A and B. Enzyme activity is measured in units of 10^{-6} nmol/10min/cell.*

Nitrite Concentration in the Medium. Series A and B were similar only in regard to large amounts of nitrite detected on the first day of the assay (Figure 12). There was a decrease in the concentration to day 3 in Series A, and day 4 in Series B. There is much variability in the amounts of nitrite present in the medium in Experiment 4A and 4B (Figure 13). However, on day 1, there is little difference between the two experiments. On day 1, the concentration of nitrite is 1.18 x 10^{-6} nmol/ml in Experiment 4A; it is 1.31 x 10^{-6} nmol/ml in Experiment 4B. From day 4-6, the concentration increases to a value of 0.85 x 10^{-6} nmol/ml in Experiment 4A, whereas, in Experiment 4B, nitrite concentration decreases after day 5 to a value of 0.04 x 10^{-6} nmol/ml.

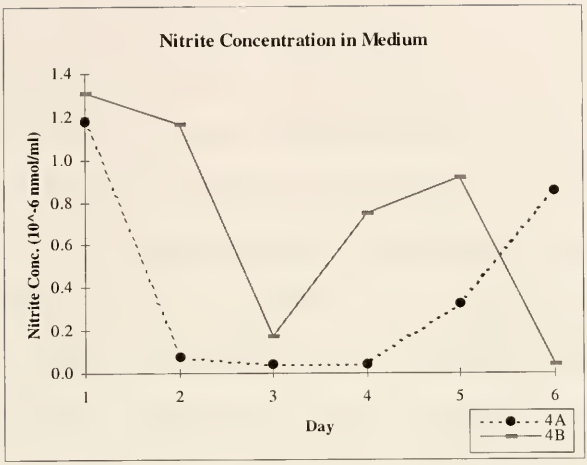


Figure 12: *Amount of nitrite present in the medium in Experiment 4A and 4B. Nitrite concentration is measured in units of 10^{-6} nmol/ml.*

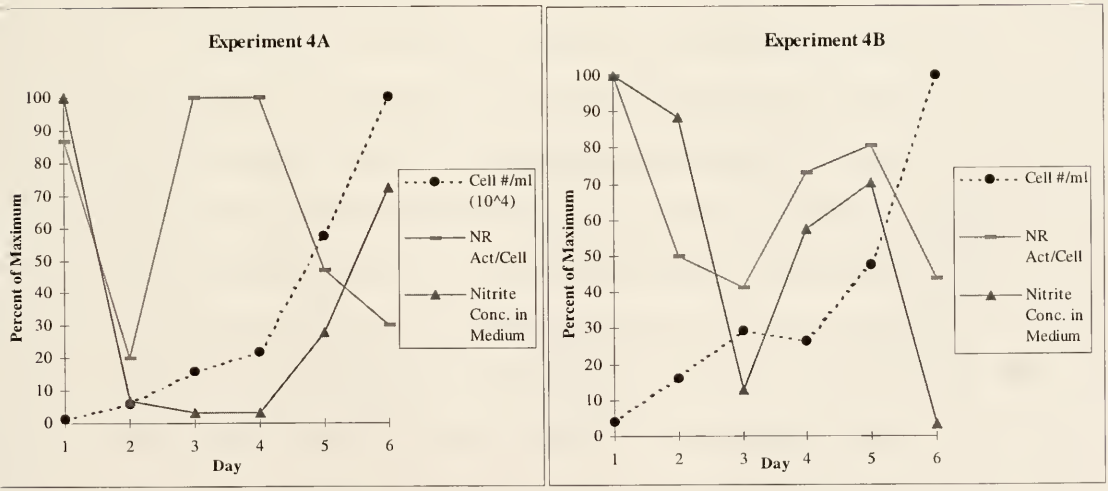


Figure 13: *Comparison of composite growth curves (10^4cells/ml), NR activity per cell (10^{-6} nmol/10min/cell), and the amount of nitrite present in medium (10^{-6} nmol/ml) in Experiment 4A and 4B. These values have been adjusted so that 100% represents its maximum value.*

Experiment 5:

In this experiment, modifications were made from the previous experiment in order to eliminate variability. Two sets of single flasks (Series A and B) were used. Each series consisted of 9 experimental flasks that were inoculated with 0.25ml from a single 5-day-old stock culture. From each of these duplicate flasks (Series B was designated as duplicates of Series A), cell number was determined daily. On each subsequent day of the experiment, a different flask was assayed for cell number, NR activity, and nitrite concentration in medium. In addition to the 18 flasks being assayed, 6 extra flasks were maintained solely for observation of cell number throughout the experiment. All 24 flasks were rotated daily to make certain that each received similar light intensities. The cultures were monitored for 9 days.

Culture growth. Cell number was determined daily in all flasks. Culture growth was observed in the six single control flasks. Composite growth curves for Series A and B were also determined (Figure 14). Both single and composite growth curves were very similar in number, with the exception of Flasks 6A and 6B. In the composite growth curves, the lag phase lasted to day 3. Thereafter, the log phase continued to day 6. By day 7, cell number began to fall off rapidly. Cell debris was observed in the culture medium after day 6, suggesting the culture had reached its death or decline phase. Cell number ranged from 818 x 10^4 cells/ml (5A) to 983 x 10^4 cells/ml (5B) on day 6; from 350 x 10^4 cells/ml (5A) to 368 x 10^4 cells/ml (5B) on day 9 in the flasks used for the composite growth curves.

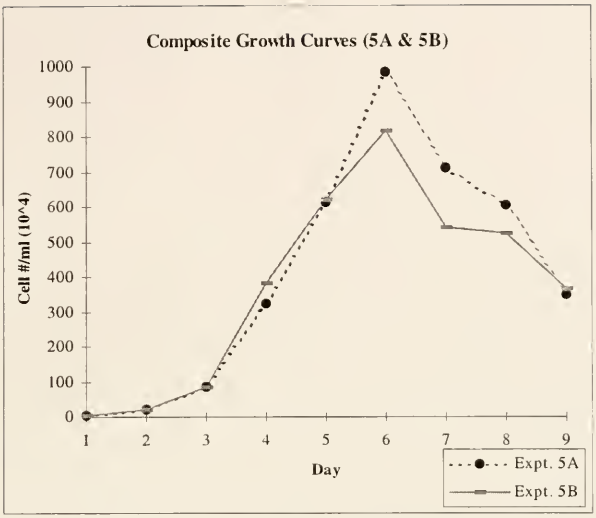
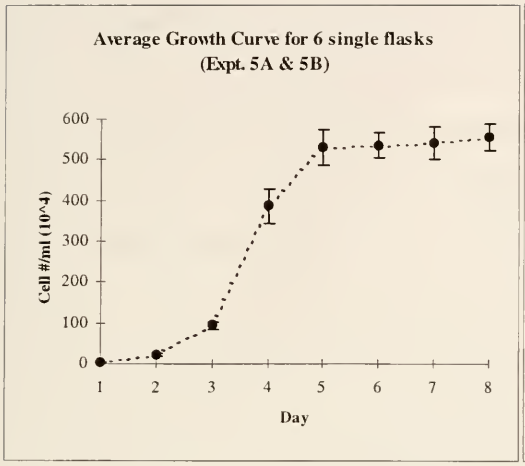


Figure 14: *Composite growth curves from Series A and B of Experiment 5. Cell number per milliliter (10^4) was calculated daily in each assayed flask.*

The growth curves of six single culture flasks are extremely similar (Figure 15). The growth curves appear to remain in the lag phase to day 3. The log phase begins on day 3 and lasts to day 5, where stationary phase begins and continues to the end of the experiment (day 9). Also, the growth curves of Flasks 6A and 6B have been plotted against the typical growth curve to show the difference between these two flasks with the six control flasks. Flask 6A and 6B seem to be the only flasks which deviate from the typical growth curve. These flasks happened to be the flasks that were assayed on day 6 for enzyme activity. Flasks 6A and 6B reach an average cell number of 901 x 10^4 cells/ml on day 6 in comparison to a cell number of 536 x 10^4 cells/ml in the 6 control flasks.



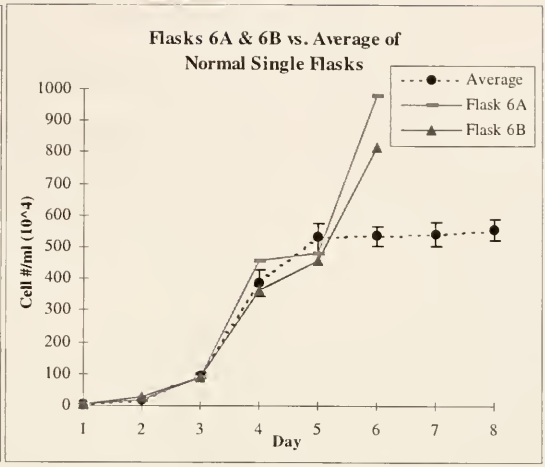


Figure 15: *Average growth curve for 6 single control flasks versus Day of assay in comparison to Flasks 6A and 6B from the composite growth curves. Cell number (10^4) per milliliter were determined daily. Error bars represent one standard deviation.*

NR activity. Series A and B from Experiment 5 were almost identical in that NR activity decreased dramatically after day 2 (Figure 16) during the logarithmic phase of growth. The greatest variability in NR activity per cell was observed in the first 2 days. Series 5B had higher levels of enzyme activity on these days in comparison to Series 5A. On day 4 of both series, negligible levels were detected. In Experiment 5A and 5B (Figures 17), NR activity per cell and growth were monitored for 9 days. The enzyme activity per cell decreased as the cells entered the logarithmic phase of growth and stayed relatively constant after day 4. Even when the cells peak at stationary phase, there is little change in enzyme activity. However, there seems to be a small blip in the activity on day 8. The pattern to take note of in these experiments is the low amounts of enzyme activity from day 4 to the last day of the assay. In these two experiments, the NR activity per cell

ranged from 1.75 x 10^{-6} nmol/10min/cell to 2.00 x 10^{-6} nmol/10min/cell on day 1, from 0.07 x 10^{-6} nmol/10min/cell to 0.138 x 10^{-6} nmol/cell on day 4, and from 0.032 x 10^{-6} nmol/10min/cell to 0.054 x 10^{-6} nmol/10min/cell on day 9. In comparison to NR activity per cell in the previous experiment, minimal variation was observed in the enzyme activity between series.

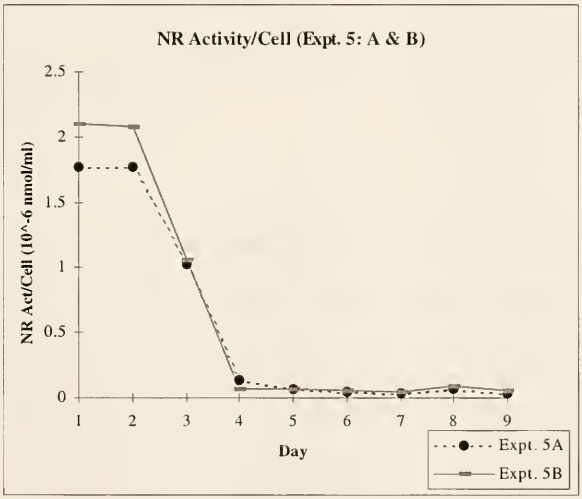


Figure 16: *NR activity per cell from Experiment 5, Series A and B. Enzyme activity was measured in units of 10^{-6} nmol/10min/cell.*

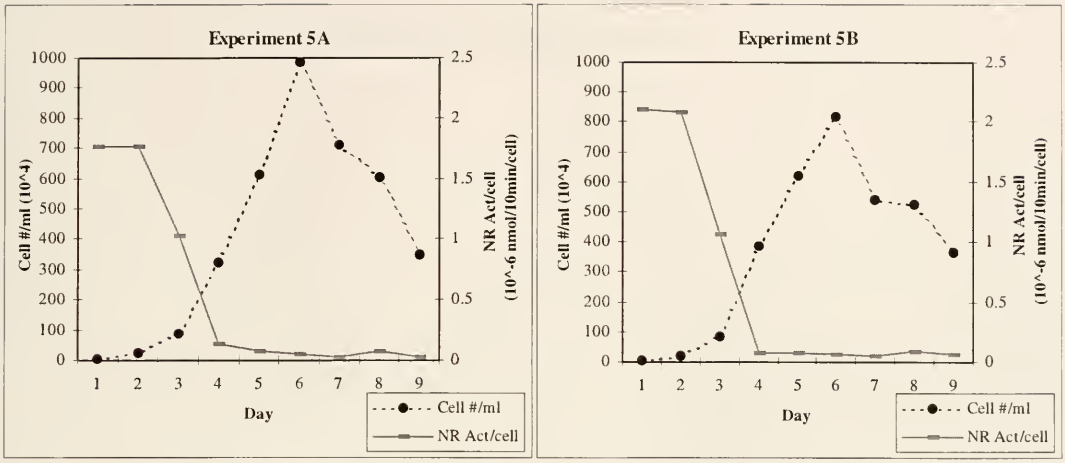


Figure 17: *Composite growth curves and NR activity per cell in Experiment 5. Cell number per cell was measured in units of 10^4 cells/ml. NR activity/cell was measured in units of 10^{-6} nmol/10min/cell.*

Nitrite Concentration in the Medium. The levels of nitrite in the medium in Experiments 5A and 5B exhibit very similar patterns. Minimal amounts of nitrite were present in the medium until day 4. Beginning on day 4, the amounts of nitrite increased until day 6 in Experiment 5B and day 7 in Experiment 5A (Figures 18). Thereafter, concentrations decreased until day 9. In Experiment 5A, the concentration of nitrite in the medium increased to 49.5 x 10^{-6} nmol/ml, whereas in Experiment 5B it only reached a concentration of 22.5 x 10^{-6} nmol/ml.

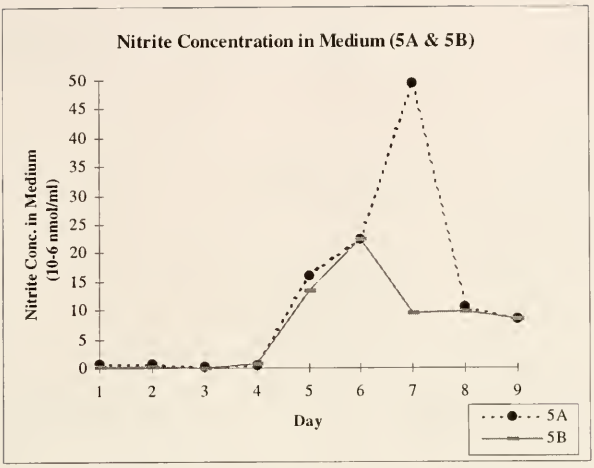


Figure 18: *Amount of nitrite present in the medium versus day of assay in Experiment 5. Nitrite concentration was measured in 10^{-6} nmol//ml.*

When comparing graphs of composite growth curves, NR activity per cell, and the presence of nitrite in the medium in Experiment 5, several trends become evident (Figures 19). Between day 3 and day 4, during the log phase of growth, NR activity per cell decreases to 20% of its maximum value. Cell number and the amount of nitrite in the medium appear to follow similar patterns and decrease around day 6. On day 4, enzyme activity decreased to 10% or less of its maximum value, and the amount of nitrite present in the medium increased. With the exception of the amount of nitrite present in the medium on day 7, there was little variability between Series A and B of this experiment. This indicates that the precautions taken to decrease variability were effective.

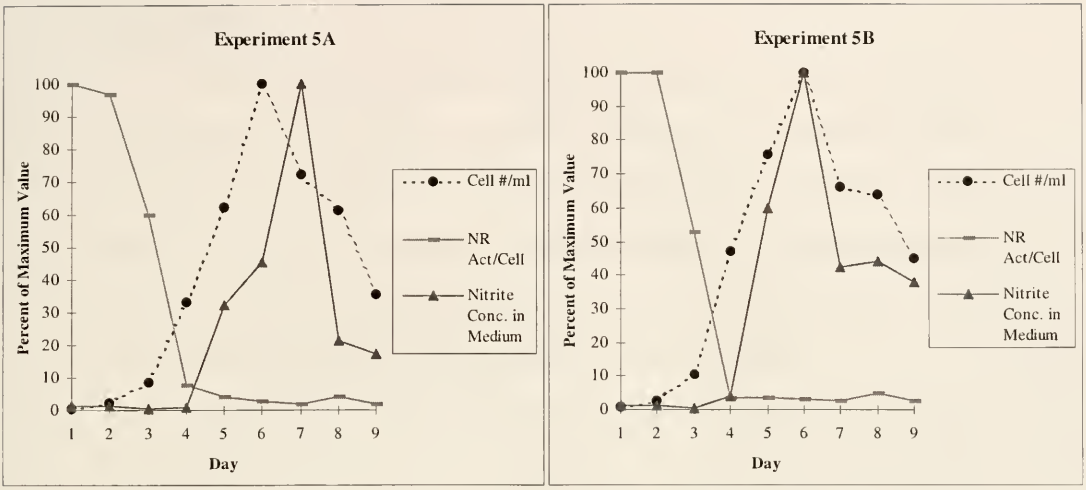


Figure 19: *Comparison of composite growth curves (10^4 cells/ml), NR activity per cell (10^{-6} nmol/10min/cell), and the amount of nitrite present in the medium (10^{-6} nmol/ml) in Experiment 5. These values have all been adjusted so that 100% represents its maximum value.*

Experiment 6:

In this preliminary experiment, three sets of 9 culture flasks were used. Each set was labeled as either 0x acetate, ½x acetate, or 2x acetate. All 27 experimental flasks were inoculated with 0.25ml from a single 10-day-old stock culture. The difference in the three sets of flasks was the amount of acetate in the medium: 0x acetate contained no acetate, ½x acetate contained half (1g/l) of the usual amount (2g/l) of acetate, and 2x acetate contained twice (4g/l) the usual amount of acetate in the medium.

Composite growth curves, single flask growth curves, NR activity, and the amount of nitrite in the medium were determined. In this preliminary experiment, flasks were tested daily from day 3 through day 8. All 27 of the flasks were rotated daily to make certain that each received similar light intensities. Changes in acetate concentration affected cell number, NR activity, and the presence of nitrite in the medium.

Culture growth. Acetate concentrations affected cell number (Figure 20). In comparison to the 0x and 2x acetate, the ½x acetate treated cells had a very rapid culture growth. The lag phase of growth was relatively short, and by day 6, the cultures were in stationary phase. In the 2x flasks, the cells remained in lag phase to day 5. The log phase then began, whereas, in the 0x acetate treated cultures, the cells appear to remain in lag phase throughout the experiment.

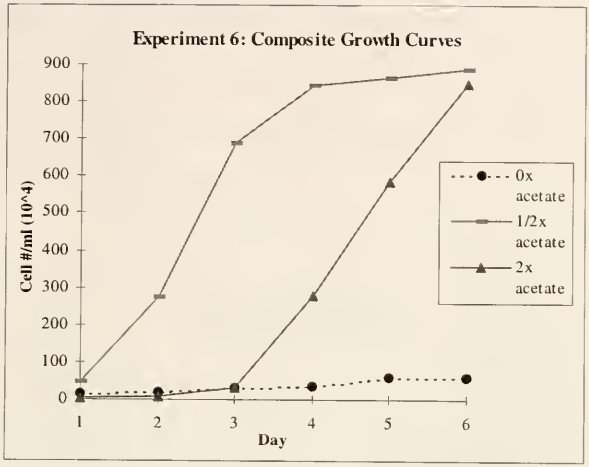


Figure 20: *Effects of varied acetate concentrations in Experiment 6 on composite growth curves. Cell number is expressed as 10^4 cells/ml.*

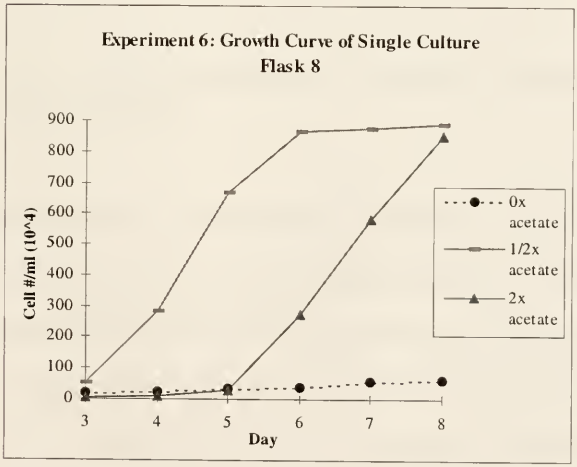


Figure 21: *Effects of varied nitrite concentrations on culture growth of a single flask in Experiment 6. Flask 8 was used as the control culture growth flask in this Experiment.*

NR activity. In the 0x flasks, there is a slower decrease of activity per cell over time than in the ½x cultures (Figure 21). However, the ½x cultures show almost negligible enzyme activity by day 5. A peak is evident on day 5 in the 2x cultures. This peak may be due to experimental error.

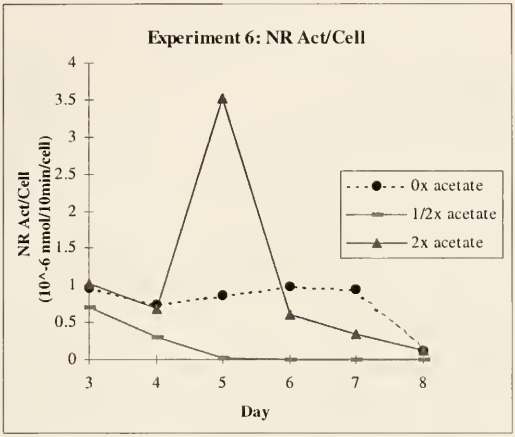


Figure 22: *Effect of acetate concentrations on NR activity. NR activity per cell was expressed in 10^{-6} nmol/10min/cell.*

Nitrite Concentration in the Medium. The amounts of nitrite present in the medium was greatly affected by acetate concentration (Figure 22). Until day 4, all the treatments displayed minimal amounts of nitrite in the medium. After day 4, the cultures without acetate showed almost insignificant amounts of nitrite in comparison to the other cultures. This is most likely due to the very low cell numbers in the cultures. The 2x culture showed small traces of nitrite in the medium until day 7 when the concentration quickly rose. In the ½x acetate cultures, there was little nitrite present in the medium

until day 4; the concentration increased linearly thereafter. After day 7, there was a slight decrease in the concentration.

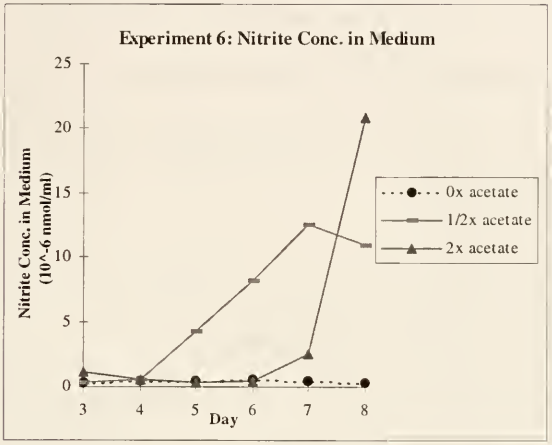


Figure 23: *Effect of acetate concentration on the presence of nitrite in the medium. Concentrations of nitrite were expressed as 10^{-6} nmol/ml.*

0x Acetate. The relationships between culture growth, NR activity per cell, and nitrite concentration in the medium in the cultures without in acetate are apparent (Figure 23). As cell number increased the enzyme activity was constant until day 7, when it decreased. The presence of nitrite in the medium was fairly constant; a small decrease is seen after day 7. On day 7, cell number increases to 90% of its maximum value while NR activity per cell and the amount of nitrite in the medium decreased.

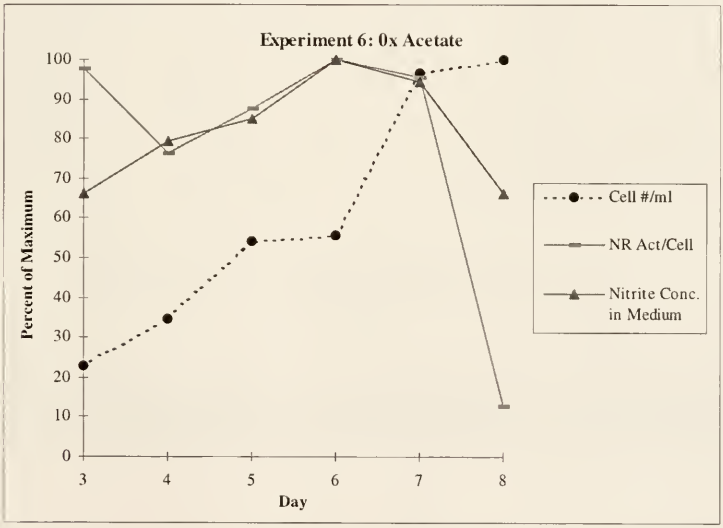


Figure 24: *Effect of 0x acetate on composite growth curves (10^4 cells/ml), NR activity per cell (10^{-6} nmol/10ml/cell), and amount of nitrite present in the medium (10^{-6} nmol/ml). These values have been adjusted so that 100% represents maximum value.*

½x Acetate. In the ½x acetate set of experimental flasks, after day 3, the culture growth is in logarithmic phase until day 6 (Figure 25). Stationary phase then began and continued to the end of the experiment. When cell number had increased to about 35% of

maximum (day 4), the enzyme activity per cell decreased. As the enzyme activity per cell decreased, nitrite in the medium increased, reaching its maximum value two days after NR activity had decreased to very low levels.

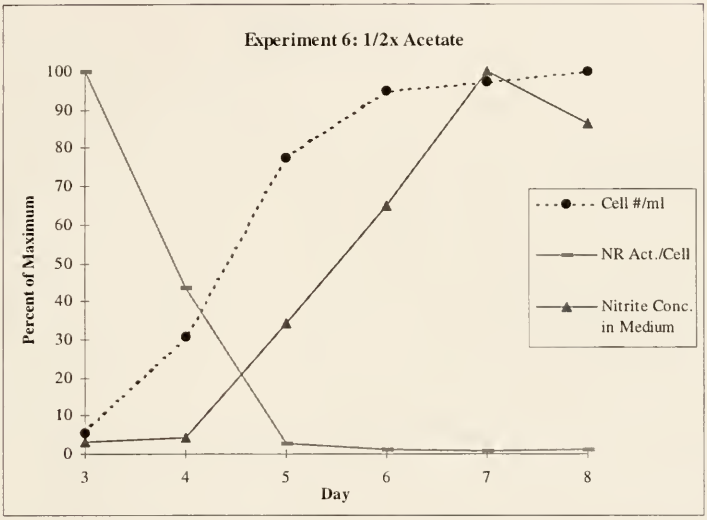


Figure 25: *Effect of ½x acetate on composite growth curves (10^4 cells/ml), NR activity per cell (10^{-6} nmol/10min/cell), and amount of nitrite present in the medium (10^{-6} nmol/ml). These values have been adjusted so that 100% represents maximum value.*

2x Acetate. In the 2x acetate set of experimental flasks, cell number and the amount of nitrite present in the medium increased, while NR activity per cell decreased. NR activity per cell was at 30% of its maximum value on day 3, increased until day 5, then decreased to day 8. Since the increase on day 5 represents only one data point, it is not clear whether the increase is real. The nitrite in the medium did not appear until day 7

when it was about 10% of its maximum value. In comparison with the other cultures, it took 3 days longer for nitrite to appear in the medium. This supports the suggestion that the added acetate supplies extra carbon skeletons to which the ammonium can bind.

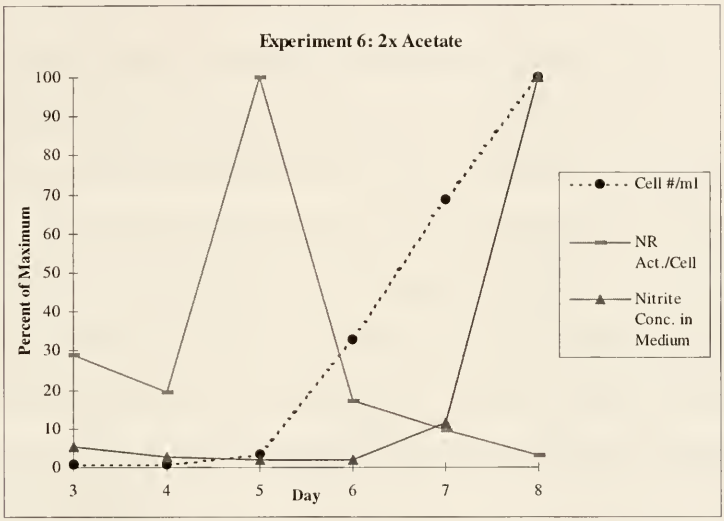


Figure 26: *Effect of 2x acetate on composite growth curves (10^4 cells/ml), NR activity per cell (10^{-6} nmol/10min/cell), and amount of nitrite present in the medium (10^{-6} nmol/ml). These values have been adjusted so that 100% represents its maximum value.*

Discussion

Short-term changes in NR activity in *Chlamydomonas reinhardtii* have been studied by a number of researchers. Hipkin, et al. (1980), and Azuara and Aparicio (1983, 1984), studied changes in NR activity for up to 6 hours after placing the cells in a nitrate-containing medium. In the experiment presented here, cells were grown in a nitrate-containing medium and assayed for NR activity on long-term basis; the enzyme was monitored on a per cell basis every 24 hours for periods of 6 to 9 days.

My experiments demonstrate that NR activity per cell is not constant during the growth of *Chlamydomonas reinhartii.* My original hypothesis stated that enzyme activity per cell would remain constant; however, the changes that could occur in the medium during the growth of the culture were not taken into account. Possible reasons for the differences in enzyme activity per cell include: 1) a decrease in nitrate concentration, 2) an increase in nitrite or ammonium in the medium, and 3) a decrease in the ability of the cells to take up nitrate.

A decrease in the amount of substrate in the medium would result in a reduction of enzyme activity per cell. Levels of nitrate in the medium during culture growth were not determined experimentally. It was however assumed that due to the constant need for nitrate by the cells, there was a decrease in the concentrations of nitrate. Azuara and Aparicio (1983) postulated that low CO_2 tensions result in the excretion of nitrite and ammonium by the cells. It can, therefore, be assumed that ammonium is present in the medium. It is unknown, however, whether or not nitrate levels in the medium, or nitrate consumption decreased due to the presence of ammonium.

Hipkin et al. (1980), stated that ammonium inhibits nitrate uptake, and that NR activity is low or negligible during ammonium's presence. A decrease in the cell's ability to consume nitrate would cause a change in the levels of enzyme activity during culture growth. During nitrogen assimilation, there is a lack of carbon skeletons in the medium. The ammonium is left without anything on which to attach resulting in the excretion of nitrite and ammonium by the cell. Azuara and Aparicio (1983) also observed that after the addition of ammonium to cells grown on nitrate, most of NR is present but in its inactive form. It is possible that the negligible levels of NR detected in my experiments are in fact inactive NR.

In addition to Azuara and Aparicio's observations, Hipkin et al., also observed that small increases in NR activity were seen in nitrogen-starved cells for up to two hours; subsequently, the enzyme activity fell to negligible levels (Hipkin et al., 1980). This suggests that without the presence of a nitrogen source, specifically nitrate, the enzyme become inactive after two hours. This reinforces the substrate inducibility of NR.

When referring to Figures 7, 13 (Experiment 4A), and 19, a drastic drop in enzyme activity and a large increase in nitrite concentration were seen before the cells entered the logarithmic phase. As previously mentioned, the excretion of nitrite and ammonium could inhibit nitrate uptake by the cells. The conversion of active NR to an inactive form of NR could be occurring after a few days of culture growth. This conversion is a result of high amounts of ammonium in the medium.

In my experiments, carbon dioxide in the medium may become limiting as the cultures grow dense. This would limit photosynthesis and results in a decrease in the number of available carbon skeletons. NR activity decreased after day 3 (Figures 7, 13,

19) falling to undetectable levels by day 4. On this same day, nitrite concentration in the medium increases drastically (Figures 19). Hipkin et al. (1980) conclude that nitrite and ammonium are excreted simultaneously. It can, therefore, be suggested that the presence of ammonium in the medium is inhibiting nitrate consumption, which, in turn, causes a dramatic drop in enzyme activity and the drastic increase in the presence of nitrite.

A decrease in nitrate concentration, a decrease in the cells ability to assimilate nitrate, and an increase in the presence of nitrite and ammonium in the medium, are all affected by cell density. It has been observed by others that an increase in cell density causes a decrease in nitrate consumption. Azuara and Aparicio (1984) have shown that nitrate consumption and the release of nitrite and ammonium are dependent on the cell densities in the suspensions. They found that when the cell density reaches a certain range (40 to 10 $\mu g\ Chl \cdot ml^{-1}$), nitrate consumption decreased by a factor of two. In addition, nitrite excretion dropped by an order of magnitude; the excretion of ammonium was at an insignificant level (Azuara and Aparicio, 1983).

When comparing experiment performed by Azuara and Aparicio to my own, differences of techniques used must be taken into account. In my experiment, whole cells were counted for determination of cell number, whereas, Azuara and Aparicio used chlorophyll determination as their method of counting cells. In order to reach a cell count, Azuara and Aparicio assume that each cell contains equal amounts of chlorophyll. Since it is not certain that the levels of chlorophyll are equal in all cells, this method in obtaining their data is questionable.

According to my results, one can hypothesize that nitrate consumption decreased. In contrast to the work of Azuara and Aparicio, the decrease in the excretion of nitrite

was not seen; instead, a large increase was observed. An increase in cell density blocks the ability of the cells to consume nitrate. This can be seen in the experiments presented here. In Figures 5, 11, and 17, cell number increases as it enters its log phase on day 3, NR activity begins to decrease. A decrease in enzyme activity suggests that the cell is having difficulty absorbing nitrate.

Light intensity also has an affect on cell number. In Experiment 4, light intensity was measured after the experiment was completed. It was found that although all cultures were grown under the same lights, there was a considerable difference in culture growth on day 3-6 (Figures 8 and 9). This difference was due to the fact that the Series A flasks were in a slightly different position and were thus unintentionally exposed to twice the irradiance as the Series B flasks. After day 3, the series that was exposed to twice the irradiance (32 μmol s^{-1} m^{-2}) had twice the amount of cells present at the end of the experiment than Series 4B which was exposed to 17 μmol s^{-1} m^{-2}. On the last day of the assay, cell number for Experiment 4B was 99 x 10^4 cells/ml in comparison to 213 x 10^4 cells/ml. The difference in irradiance greatly affected cell number. That, in turn, affected the ability to accurately compare NR activity and the amount of nitrite in the medium between Series A and Series B.

Azuara and Aparicio (1984) also observed that for algal cells growing under high irradiances and under low carbon dioxide tensions, nitrate becomes blocked from entering the cell. As suggested in the works by Azuara and Aparicio, under low CO_2 tensions, nitrite and ammonium are excreted by the cell into the medium. Since my experimental cultures were not kept on a shaker during the experiments, it can be expected that the CO_2 levels were not evenly dispersed throughout the culture. Most of the cells closer to the

base of the flask were deprived of carbon dioxide. Again, this insufficiency in deprivation of carbon skeletons leads to the release of nitrite and ammonium. As evident in Figures 4, 10, and 16, the presence of ammonium is another possible explanation for the low amounts of NR activity detected after day 4 in the experiment.

Hipkin et al. (1980) studied how nitrate and ammonium affected NR activity in mutant *Chlamydomonas* cells. They experimented with how the addition of nitrate and ammonium to cells pre-grown on ammonium, washed with nitrogen-free medium, and resuspended in nitrate or nitrogen-free medium affected the enzyme activity for up to 6 hours (48 hours in one experiment). In these experiments, Hipkin et al. (1980) showed that when ammonium-grown *Chlamydomonas* cells were resuspended in nitrate medium, NR became active and increased in cell number for up to 4 hours. Ammonium causes the enzyme to become inactive until the ammonium is removed from the medium or taken back into the cell. This is evidence for ammonium inhibiting nitrate induction in my experiments.

In Experiment 5 (Figure 18), the decline in the presence of nitrite detected in the medium after day 7 is puzzling. It is uncertain where the nitrite went. Without chemical oxidation of nitrite back to nitrate, it is not possible for nitrite to simply disappear from the medium unless nitrite consumption is occurring by the cells.

In a series of six experiments, variability was seen in cell number, enzyme activity, and presence of nitrite in the medium. In Experiment 5, however, modifications were made which resulted in low variability.

In culture growth (Figures 5, 11, and 17), the cells are not very dense at this point. It is, therefore, intriguing as to why nitrate consumption is being blocked. If an increase in

cell density affects the decrease in NR activity, then enzyme activity is decreasing earlier than expected. The enzyme activity is decreases right as the cells enter their log phase. This raises the question of the reliability of the differences. The factors which may affect these differences include: 1) permeabilization of the cell, 2) age of stock culture, and 3) number of cells in inoculate.

In testing for NR activity, a problem in the addition of nystatin during the permeabilization of the cell membrane could attribute to a decrease in enzyme activity per cell after day 3. It is possible that the 50µl of antibiotic added to a 10ml volume of cells is insufficient to permeabilize all of the cells after day 3. It was thought that the cell density was too large and the nystatin was not reaching the membranes of all of the cells. This was in fact tested and disproved. To test this, 5ml and 10ml of culture were assayed in the same manner each with the addition of 50µl nystatin and the absorbance value of the 5ml aliquot was exactly half that of the 10ml aliquot. This suggests that an insufficient amount of nystatin is not the cause of the variations seen in the enzyme activity.

From experiment to experiment, the age of the stock culture in which the inoculates for the experimental cultures were taken, varied. This variation accounted for the differences observed in the enzyme activity per cell. The inoculates taken for Experiments 1-3 were taken from a 7-day-old stock culture, Experiment 4A and 4B were started from 15-day-old stock cultures, and the inoculates for Experiment 5A and 5B were taken from a 5-day old stock culture. In the first few days, variation should have been expected due to any medium carried over in the inoculate into the experimental

flasks. This variation affected the enzyme activity per cell and the presence of nitrite in the medium on the first few days of testing.

A large variation can be noted in the presence of nitrite in the medium in Experiment 4A and 4B due to culture age. On the first day of Experiment 4 and the first two days of Experiment 4A, there is a greater amount of nitrite present; this was most likely due to the 15-day-old stock culture. Although the medium of a 15-day-old stock culture had not been assayed for levels of nitrite, it is possible that there was a large amount of nitrite present on day 15. Any amount of nitrite carried over in the inoculate will be detected in the first few days of the enzyme assay.

It is possible, however, that the difference in age of stock cultures could have been a variable explaining the inconsistencies in cell number and in NR activity patterns. As noted in the results, the levels of enzyme activity differed both within and among experiments; the activity patterns in the experiment lasting to day 6 differs from the activity pattern in the experiment lasting to day 9. This could be due to differences of the stock culture ages used in the experiments.

All of the growth cultures were started with the same volume of stock culture, however, it was not certain if each volume contained the same number of cells. The cultures in the experiments presented here were assayed daily for a period of six days and the same cell number was never reached among all experiments. On day 6 of the assay, cell number ranged from 99 x 10^4 cells/ml to 2000 x 10^4 cells/ml. It is possible that the reason NR activity was greater on day 4 of Experiment 1, than on day 4 of Experiment 3 is that in Experiment 1, fewer cells were present.

Experiment 6 was set up to test the hypothesis which stated that the adding of organic carbon sources to the medium, there would be a smaller decrease in the presence of nitrite in the medium. There would have also been an increase in enzyme activity per cell due to the binding of ammonium to the organic carbon skeletons.

The organic carbon source, acetate, appears to have affected the growth of the cultures greatly. The cultures which were grown on a medium without an organic carbon source experienced minimal growth. The cultures exposed to twice the acetate remained in log phase until the end of growth, and those exposed to half the amount of acetate quickly reached stationary phase on day 6. Previous experiment observed that with half the amount of acetate, the cells remained in lag phase until day 5. Those exposed to twice the amount of acetate, reached stationary phase by day 6. In the earlier experiments where the cultures were grown on 1x acetate, the stationary phase lasted only one day and then an immediate decrease was observed. This preliminary experiment suggests that an increased concentration of acetate has a large affect on culture growth.

In observing the enzyme activity per cell, I found that in relation to cell number, the cells exposed to twice the acetate contained a higher amount of NR activity than the 1/2x acetate. This difference suggests that the added carbon skeletons were binding to the ammonium preventing the nitrite and ammonium from being excreted. Since this was a preliminary experiment and duplicate flasks were not set up, as in previous experiments, these results are somewhat ambiguous. Further testing is necessary in order to determine how various levels of concentrations of organic carbon sources directly affect NR activity.

The cells grown on the 2x acetate did not excrete nitrite into the medium until day 7. This would suggest that in order to provide sufficient carbon skeletons until day 7, additional carbon sources must be added to the medium. This is necessary for the ammonium to bind forming organic-nitrogen compounds. By adding acetate, the ammonium remains bound to carbon skeletons for a longer period of time as a result of nitrogen assimilation. It is, therefore, evident that the acetate is required for a decreased release of nitrite and ammonium. In turn, this sustains NR activity for a longer period of time. Once again, this experiment was preliminary.

It remains uncertain whether NR activity is greatly affected by the addition of an organic carbon source. It can be hypothesized, however, that if nitrite was not excreted into the medium until day 7, that more NR activity per cell would have been present. Further testing with carbon sources will be done in order to determine that validity of this hypothesis. Duplicate flasks will be necessary to ascertain variations in carbon concentrations affect NR activity per cell and the presence of nitrite in the medium.

Differences in experimental set-up can account for variability of experiments. Due to variables, it is difficult to compare the results of these experiments. Patterns in the data, such as, increases or decreases, can be observed. Quantitative values can only be compared within an experiment. For instance, in Experiment 5, the values from Series A can be compared with those from Series B. Experiment 1 and Experiment 4 are incomparable due to the fact the set-up of the two experiments differed.

The research introduced here sheds new light on future experiments. The goal of future research should be to eliminate as much variability among experiments as possible. To accomplish this, 1) duplicate flasks will always be used, 2) experimental cultures will

be maintained on a shaker, 3) more attention should be paid to consistency of light intensity, and 4) all experimental cultures will be inoculated with equal numbers of cells.

In determining cell number, extra flasks should be kept as controls in addition to the experimental flasks, in case a problem arises with the cell number in the experimental flasks. This problem developed on day 6 of Experiment 5 (Figure 15). Control flasks will allow for the flask that contains an unusual value to be discarded. One of the control flasks could then be assayed. Moreover, experimental cultures should be maintained on a shaker in order to evenly distribute carbon dioxide levels throughout the culture. It would be interesting to compare growth curves, NR activity, and presence of nitrite in the culture medium maintained on a shaker as opposed to unshaken.

In inoculating experimental flasks with stock cultures, stocks of the same age should be used. More importantly, cell number of each inoculate must be identical. Cell number can be regulated by, first, determining the cell number per milliliter of the stock culture used. The inoculate can then be diluted with media so that all inoculates are equal in number and volume. By regulating cell number during culture growth among experiments, it is possible to compare and contrast experiments.

In determining the amount of nitrite present in the medium, intracellular nitrite levels should be compared to extracellular nitrite levels during culture growth. If the sum of intracellular and extracellular levels is constant throughout culture growth, one might deduce that each cell produces equal amounts of nitrite, or that NR activity is equal in all cells. If the sum of the two amounts of nitrite are not equal in all cells during culture growth, it would support my hypothesis that NR activity is not constant throughout the growth of the culture.

By testing levels of nitrate and ammonium in the medium, one could explain whether or not the excretion of ammonium affects the consumption of nitrate by the cells. One could also compare levels of excreted ammonium and nitrite to determine whether equal amounts are released. One could also monitor levels of organic carbon sources in the medium in a set of flasks which could then be compared to cultures administered with similar concentration of carbon dioxide.

Conclusion

My research has shown that NR activity in *Chlamydomonas reinhardtii* experiences daily variations. It has been determined by assaying for the release of nitrite by the cells, that NR activity per cell is greatest during the first two days of culture growth. From this, I now know when NR activity is at its highest and, therefore, when is best to assay for NR. I am now able to apply these results to more extensive research in hopes of detecting NR in other unicellular organisms.

Taking such precautions and testing various factors, as previously suggested, will minimize variability among experiments. By better understanding the effect of culture conditions on NR activity in *Chlamydomonas reinhardtii,* future researchers should find this information useful when looking for the presence of NR in other unicellular organisms.

Appendices

Appendix A:

Ingredients of Sager-Granick Nitrate medium II (Reiss, 1994). (1000ml)

Compound	*Amount Added*
NaH_2PO_4	3.67g
K_2HPO_4	1.15g
Na acetate	2.00g
5% Na citrate	10ml
0.1% $FeCl_3$	10ml
0.4% $CaCl_2 \cdot 2H_2O$	10ml
1.5% $MgSO_4$	10ml
10% KNO_3	10ml
"Trace element stock"	10ml

*0.1% $FeCl_3$ must be freshly made.

Trace element Stock	*Amount Added (mg)*
H_3BO_3	100
$ZnSO_4 \cdot 7H_2O$	100
$MnSO_4 \cdot 4H_2O$	40
$CoCl_2 \cdot 6H_2O$	20
$Na_2MoO_4 \cdot 2H_2O$	20
$CuSO_4$	4

Appendix B

Ingredients of *Euglena gracilis* defined growth medium (Dunn and Arditti, 1961) (1000ml)

Compound	*Concentration*
KH_2PO_4	0.5 g/l
$MgSO_4 \cdot 7H_2O$	0.5 g/l
L-Glutamic acid	5.0 g/l
DL-Malic acid	1.0 g/l
$CaCO_3$	0.1 g/l
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.01 g/l
Thiamine HCl	0.001 g/l
Cyanobalamin (Vit. B_{12})	1 x 10-6
"Metals 36"	10 ml/l

Metals 36 compound	*Concentration (g/l)*
$MnSO_4 \cdot 7H_2O$	6.15
EDTA	5.0
$ZnSO_4 \cdot 7H_2O$	11.0
$FeSO_4(NH_4)_2SO_4 \cdot 16H_2O$	1.75
$CoSO_4 \cdot 7H_2O$	0.286
$CuSO_4 \cdot 5H_2O$	0.039
H_3BO_3	0.028
KI	0.0012

Appendix C

Ingredients of undefined enriched growth medium (ATCC 1338) (1000ml)

Compound	*Amount Added*
Na acetate (anhydrous)	2.0 g
Bacto-tryptone	2.0 g
Yeast extract	2.0 g
Bacto-agar	15.0 g
dH_2O	1.0 L

Appendix D

Ingredients of defined heterotrophic growth medium. (1000ml)

Compounds	*Concentration (g/l)*
KNO_3	1.25
KH_2PO_4	1.25
$MgSO_4 \cdot 7H2O$	1.00
$CaCl_3$	0.0835
H_3BO_3	0.1142
$FeSO_4 \cdot 7H_2O$	0.0498
$ZnSO_4 \cdot 7H_2O$	0.0882
$MnCl_2 \cdot 4H_2O$	0.0144
$Na_2MoO_4 \cdot 2H_2O$	0.0071
$CuSO_4 \cdot 5H_2O$	0.0157
$Co(NO_3)_2 \cdot 6H_2O$	0.0049
EDTA	0.5

Appendix E: Solutions for enzymatic assay.

Nitrate buffer (pH 7.1): 250mM $NaH_2PO_4 \cdot H_2O$ (6.9g/200ml), 50mM $NaNO_3$ (0.85/200ml), 50µM dithiothreitol (DTT): 1ml of 10mM DTT (0.15g/100ml). Check pH and adjust if necessary.

Nystatin (5000units/ml): 5mg nystatin/5ml distilled H_2O. Made fresh and stored on ice.

1% Sulfanilamide: 4g/400ml of a solution of 100ml concentrated HCL and 300ml distilled H_2O.

0.2% N-1-naphthylethylenediamine (NEED): 0.08g/400ml distilled H_2O

Nitrite standard solution (25nmol/ml): 85mg KNO_2/100ml solution made first. 10mM nitrite stock solution made with 1ml of the KNO_2 solution added to 400ml distilled H_2O.

Appendix F

Protocol for nitrite standard curve using a standard nitrite solution of 25nmol/ml (Reiss, 1994).

Tube #	Nitrite (nmol)	Nitrite Solution (ml)	H_2O (ml)
1	0	0.0	2.0
2	10	0.4	1.6
3	20	0.8	1.2
4	30	1.2	0.8
5	40	1.6	0.4
6	50	2.0	0.0

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I pledge that I will guarantee the validity of my word, maintain absolute honesty in my work, and respect the property of others. Realizing that these standards are an integral part of life at Sweet Briar, I hereby assume my obligation to uphold them. I will report myself and ask others to report themselves for any infraction of this pledge.

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